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Production and Properties of Cyanobacterial Endotoxins

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Lipopolysaccharides (LPS) were isolated from four species of cyanobacteria (*Anabaena flos-aquae* UTEX 1444, *A. cylindrica*, *Oscillatoria tenuis*, and *O. brevis*) frequently occurring in drinking-water supplies. The cyanobacterial LPS contained glucose, xylose, mannose, and rhamnose, but differed from the LPS derived from most gram-negative bacteria because of the variable presence of 2-keto-3-deoxyoctonate, heptose, galactose, and glucosamine. Cyanobacterial lipid A is characterized by long-chain saturated and unsaturated fatty acids and hydroxy fatty acids which show great diversity. Unlike lipid A from heterotrophic gram-negative bacteria, lipid A from cyanobacteria usually lacks phosphates. The detection of distinct O-antigen chemotypes indicates that LPS may be used for taxonomic classification. Isolated cyanobacterial LPS always induced *Limulus* amoebocyte lysate gelation. *A. flos-aquae* LPS gave a positive Schwartzman reaction. Endotoxins from *A. cylindrica* and *O. brevis* were toxic to mice when injected intraperitoneally. The cyanobacterial endotoxins showed generally lower biological activity than did LPS derived from common heterotrophic gram-negative bacteria. Nevertheless, cyanobacteria in algal blooms may be a significant source of endotoxins in water supplies.

Weise et al. (36) first isolated lipopolysaccharides (LPS) from *Anacystis nidulans*, and since this initial finding, endotoxins were found in several species or strains of cyanobacteria, including *Anabaena variabilis, Phormidium africanum, P. laminosum, P. uncinatum, Agmenellum quadruplicatum*, and *Schizothrix calcicola* (1, 15, 20, 35). Additionally, Weckesser et al. (34) have identified seven chemotypes of *Synechococcus* and *Synechocystis* strains (synonyms of *Aphanocapsa* and *Gleocapsa*) based on the sugar and fatty acid composition of their LPS. The taxonomy of cyanobacteria was revised by Rippka et al. (23) and new synonymy has been established for most of the species analyzed for LPS. Thus, *A. nidulans* is the synonym for *Synechococcus* PCC6301 KM, *A. quadruplicatum* for *Synechococcus* PCC73109 and *A. variabilis* became *Anabaena* PCC7118.

The LPS derived from cyanobacteria and from gram-negative bacteria, although basically similar, differ in both chemical and biological characteristics. All bacterial LPS can probably cause endotoxemia, particularly in debilitated, immunosuppressed patients. The enhanced gastrointestinal permeability of infants suggests that they are especially susceptible to exogenously derived endotoxins from milk and water (4). However, healthy populations may have been affected by LPS. For example, Rylander and Lundholm (25) suggested that bacterial endotoxins produced gastrointestinal tract disorders in workers at six sewage treatment plants. Rylander et al. (24) identified humidifier fever with the exposure to endotoxins. Muittari et al. (21) described an outbreak of bathwater fever among more than 100 people from endotoxins in tap water.

Once waterborne endotoxins were associated only with gram-negative heterotrophic bacteria. DiLuzio and Friedmann (4), Evans et al. (5), Jorgensen et al. (11, 12), and Watson et al. (33) believed that these organisms were the major source of endotoxins in water. However, there is evidence that cyanobacteria may contaminate drinking water with endotoxins. Hindman et al. (8) described a pyrogenic reaction among hemodialysis patients to endotoxin in the tap water, possibly caused by a high alga concentration in the local raw-water source occurring when algae reached maximum levels and the river flow was lowest. The pyrogenicity of the tap water correlated with the algal counts, but because bacterial counts in the raw water were not taken, endotoxins derived from heterotrophic gram-negative bacteria cannot be excluded (8). An unusually high concentration of a blue-green alga, *S. calcicola*, or endotoxin produced by this species may have brought about an epidemic of gastrointestinal waterborne illness in Sewickley, Penn., in August 1975 (14a, 18, 29, 30).

In the research reported here, four species of the autotrophic organisms *Anabaena* and *Oscillatoria* were examined for LPS. *Anabaena* spe-

cies may occur in permanent or semipermanent water bodies, such as drinking-water reservoirs, in sufficient density to form a water bloom. Some of the toxic algal water blooms observed in different parts of the world have been attributed to *Anabaena flos-aquae*, but no information is available on LPS production by these toxic strains. *Oscillatoria* species are commonly found in drinking-water systems and may form dense blooms under semianaerobic conditions in stagnant water in lakes, ponds, and reservoirs. This genus has not been subjected to endotoxin isolation and characterization.

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MATERIALS AND METHODS

Cultures of algae and cyanobacteria. *A. flos-aquae* toxic strain NRC 44-1 was obtained from Wayne W. Carmichael, Wright State University, Dayton, Ohio. *A. flos-aquae* UTEX 1444, *Oscillatoria brevis* UTEX 1567, *Oscillatoria tenuis* UTEX 1506, and *Anabaena cylindrica* UTEX 1611 were acquired from the collection of the University of Texas at Austin. *Oscillatoria tenuis* was cultured in Allen medium, and all of the other investigated organisms were cultured in ASM-1–Tricine medium (2, 9). All species were mass cultured and harvested as described by Keleti et al. (15). Cyanobacteria were counted in selected cultures. The liquid containing these organisms was preserved with Lugol solution, concentrated by sedimentation, and counted in a Palmer-Maloney nannoplankton cell. The results were expressed as the number of cells per milliliter. The heterotrophic bacterial contamination of cyanobacteria was determined by plating serial dilutions in 0.85% sterile saline of the cultures on Trypticase soy agar (BBL Microbiological Systems) in duplicate. Based on the method of Keleti et al. (15) contaminating heterotrophic bacteria in the cyanobacterial cultures constituted only 0.03% of the total biomass.

Isolation and purification of LPS. Harvested cells were washed three times with sterile distilled water, freeze-dried, and subjected to hot-phenol-water extraction. From the resulting crude LPS, glucan was removed by enzymatic hydrolysis with cellulase (15).

Analytical procedures. Neutral and amino sugars were released from the LPS by using hydrolysis with 1 N H_2SO_4 and 4 N HCl and analyzed by descending paper chromatography. For fatty acid analysis of the lipid A fraction, the LPS was hydrolyzed with 4 N HCl, and extracted by chloroform followed by methylation and petroleum ether extraction. The residue was dissolved in ethyl acetate and subjected to chromatographic and mass spectrometric analyses. In addition, LPS were analyzed for the presence of 2-keto-3-deoxyoctonate (KDO), heptoses, total carbohydrates, proteins, and phosphorus (15).

Biological activity. The lethal effect of purified endotoxin was determined by duplicate intraperitoneal injections into five 20-g Swiss-Webster female mice at concentrations ranging from 0.1 to 5 mg. Schwartzman phenomenon and *Limulus* amoebocyte lysate (LAL) tests were also performed by the techniques and procedures described by Keleti et al. (15).

For the ligated rabbit ileal (rabbit loop) assay, the basic procedure described by De and Chatterje (3) was used. Weaners weighing 1,000 to 1,500 g were not allowed food or water for 24 h, and with aseptic precautions, a midline incision about 2 inches long, just below the middle of the abdomen, was made by cutting through the muscles and peritoneum.

A 10-cm segment of small intestine, taken midway between its upper and lower ends, was isolated with two nylon ligatures. Blood vessels were carefully avoided. The LPS suspended in 3 ml of sterile phosphate-buffered, pyrogen-free saline was injected slowly into the lumen of the isolated loop. Two loops were used in a single animal, one containing the toxic substance and the other with sterile phosphate-buffered, pyrogen-free saline. The abdomen was closed in two layers with nylon thread. The entire procedure was performed using total ethyl ether anesthesia. The animal was not allowed food and water for 24 h and was sacrificed after a further 7-h period by a rapid intravenous injection of 5 ml of air into the ear vein. A careful examination was made of the isolated loop and of parts of the small intestine above and below it. The fluid contained in the loop was aspirated by a syringe, and its volume was measured in a calibrated cylinder and compared with the control loop containing phosphate-buffered, pyrogen-free saline.

RESULTS

A. flos-aquae. LPS isolated from *A. flos-aquae* UTEX 1444 constituted 0.86% of the lyophilized cells (Table 1), a yield lower than that established for *S. calcicola* and *A. variabilis*. The neutral sugars were represented by glucose, galactose, mannose, xylose, fucose, and rhamnose, and the only amino sugar detected was glucosamine (Table 2). The low concentration of KDO (0.16% of LPS) is not unusual, since LPS isolated from other species of cyanobacteria contained from 0.13 to 1.8% of KDO. It is interesting that this compound is absent from LPS isolated from *A. variabilis* and *S. calcicola* (15, 35).

Similarly, as in almost all other cyanobacteria investigated for LPS content, heptose was absent in *A. flos-aquae* LPS. The total carbohydrate content was found to be 65%, whereas *A. variabilis* LPS contained 80.3%. The absence of phosphorus from lipid A demonstrates that not only the oligosaccharide region of cyanobacterial LPS but also the structure of lipid A is different from other gram-negative bacteria. Lipid A of *A. variabilis* contained only traces of phosphorus (0.03%).

The LPS from *A. flos-aquae* contained 12.5% proteins, whereas 20% of *A. variabilis* endotoxins was composed of proteins (35).

Gas-liquid chromatography and mass spectrometry identified 14 long-chain fatty acids: myristic, pentadecanoic, palmitic, palmitoleic,

TABLE 1. Average yield of purified LPS

Cyanobacterial strain	Wt of lyophilized cyanobacteria (g)	Wt of purified LPS (g)	Percent yield of purified LPS
A. cylindrica	32.72	0.0589	0.18
A. flos-aquae UTEX 1444	18.89	0.1625	0.86
O. brevis	27.14	0.0977	0.36
O. tenuis	24.90	0.0548	0.22

heptadecanoic, stearic, oleic, linoleic, linolenic, nonadecanoic, behenic, and from the hydroxy fatty acids β-hydroxymyristic, β-hydroxypalmitic, and β-hydroxystearic (Table 3). However, only five fatty acids were isolated from *A. variabilis* (35).

The isolated and purified LPS was nontoxic to mice when injected intraperitoneally. Positive Schwartzman reaction was detected at concentrations of 40 and 80 μg of LPS. The lowest concentration of LPS causing LAL gelation was 12.5 ng. Rabbit loop assay was negative even with 500 μg of LPS (Table 4).

The toxic strain of *A. flos-aquae* NRC 44-1 which produces alkaloids and toxic peptides designated as anatoxins (1a, 30) did not yield any LPS. Only a polysaccharide containing glucose, galactose, mannose, xylose, rhamnose, glucosamine, and an unidentified sugar migrating faster than rhamnose was isolated.

***A. cylindrica* UTEX 1611.** The LPS isolated from this species constituted 0.18% of lyophilized cells and contained the neutral sugars glucose, galactose, mannose, xylose, rhamnose, and one unidentified fast-migrating sugar (probably a 3,6-dideoxyhexose), whereas the amino sugars were represented by glucosamine (Tables 1 and 2). KDO and heptose were not present in LPS from *A. cylindrica*. The protein content was found to be 15%, slightly lower than 20% isolated from *A. variabilis*.

Gas-liquid chromatography and mass spectrometry identified an unusually high number (17) of fatty acids, including myristic, pentadecanoic, palmitic, palmitoleic, margaric, heptadec-9-enoic, stearic, oleic, linoleic, nonadecanoic, arachidic, lignoceric, and from hydroxy fatty acids β-hydroxymyristic, β-hydroxypalmitic, β-hydroxypentadecanoic, β-hydroxymargaric, and β-hydroxystearic acids (Table 3).

The isolated and purified LPS from *A. cylindrica* injected intraperitoneally had a 50% lethal dose (LD_{50}) of 2.64 mg per mouse. The LAL reaction was positive at 1 ng (Table 4).

***O. brevis* and *O. tenuis*.** These are two closely related species of the *Oscillatoria* subgroup. However, *O. brevis* contained more LPS than *O. tenuis* (Table 1).

The neutral sugars isolated from the LPS of *O. brevis* included glucose, galactose, mannose, xylose, rhamnose, and two unknown fast-migrating sugars, but no amino sugars were present. *O. tenuis* LPS contained glucose, mannose, xylose, rhamnose, glucosamine, and two unknown fast-migrating sugars which had the same R_f values as those identified from *O. brevis* LPS (Table 2).

Quantitative analysis of protein, phosphorus, carbohydrates, KDO, and heptose was performed only on the LPS of *O. tenuis*. The carbohydrate content represented 58% of the purified LPS. The very small quantity of KDO (0.07%) indicated insignificant contamination by heterotrophic bacteria, since this substance is a notorious component of the LPS core derived from heterotrophic gram-negative bacteria. Heptose was detected constituting 1.12% of the LPS.

Fourteen fatty acids including lauric, myristic, pentadecanoic, palmitic, palmitoleic, heptadecanoic, stearic, oleic, linoleic, nonadecanoic, arachidic, behenic, lignoceric, and cerotic acids were identified from the LPS of *O. brevis* (Table 3). Gas chromatographic analysis showed that the dominant fatty acids were palmitic (23.5%), oleic (19.4%), and stearic acids (16.6%). It is noteworthy that hydroxy fatty acids were absent from the lipid A of *O. brevis*.

O. tenuis LPS had fewer long-chain fatty acids than the LPS from *O. brevis*. Palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acids were isolated from the LPS of *O. tenuis*. Unlike

TABLE 2. Sugar analysis of *A. flos-aquae*, *A. cylindrica*, *O. brevis*, and *O. tenuis* LPS

Cyanobacterial strain	Sugar								
	Fucose	Galactose	Glucose	Mannose	Rhamnose	Xylose	Fast-migrating sugar I	Fast-migrating sugar II	Glucosamine
A. cylindrica		+	+	+	+	+	+		+
A. flos-aquae UTEX 1444		+	+	+	+	+			+
O. brevis	+	+	+	+	+	+	+	+	
O. tenuis			+	+	+	+	+	+	+

TABLE 3. Fatty acid analysis of *A. cylindrica*, *A. flos-aquae*, *O. brevis*, and *O. tenuis* LPS

Cyanobacterial strain	Fatty acid																					
	Lauric	Myristic	Pentadecanoic	Palmitic	Palmitoleic	Heptadecanoic	Heptadec-9-enoic	Stearic	Oleic	Linoleic	Linolenic	Nonadecanoic	Arachidic	Behenic	Lignoceric	Cerotic	β-Hydroxylauric	β-Hydroxymyristic	β-Hydroxypentadecanoic	β-Hydroxymargaric	β-Hydroxypalmitic	β-Hydroxystearic
A. cylindrica		+	+	+	+	+	+	+	+	+	+	+	+		+		+	+	+	+	+	+
A. flos-aquae UTEX 1444		+	+	+	+	+		+	+	+	+	+		+				+			+	+
O. brevis	+	+	+	+	+	+		+	+	+		+	+	+	+	+						
O. tenuis				+	+		+	+	+	+	+							+			+	+

O. brevis LPS, in *O. tenuis* LPS there were three hydroxy fatty acids found in small quantities: β-hydroxymyristic (3%), β-hydroxypalmitic (trace), and β-hydroxystearic acids (5%) (Table 3).

The Schwartzman test performed with *O. tenuis* LPS was negative. The calculated LD_{50} for intraperitoneal injection of purified *O. brevis* LPS was determined to be 3.83 mg per mouse. The LAL reaction was positive for LPS isolated from both species of *Oscillatoria*. *O. tenuis* LPS showed a much stronger positive reaction than LPS of *O. brevis* (Table 4).

DISCUSSION

The chemical analysis of cyanobacterial LPS isolated from *S. calcicola*, *A. flos-aquae*, *A. cylindrica*, *O. brevis*, and *O. tenuis* has revealed similarities, especially in the sugar content, but each species or strain exhibited unique properties. For instance, *A. variabilis* LPS contains L-acofriose, whereas LPS from *A. flos-aquae* contains xylose and fucose in addition to common core sugars. *A. cylindrica* LPS is characterized by the presence of one unidentified fast-migrating sugar (probably a 3,6-dideoxyhexose) and glucosamine in addition to common core sugars and xylose.

Fatty acids of cyanobacterial LPS also show substantial variation among species. Unlike the LPS of gram-negative heterotrophic bacteria which generally do not contain unsaturated fatty acids, all examined cyanobacterial LPS contain relatively large quantities of oleic, palmitoleic, linoleic, and occasionally linolenic acids (6). The presence of KDO and heptose, two common components of the core LPS of enterobacteriaceae, is also variable in different species of cyanobacteria. The absence of phosphorus and glucosamine, characteristic components of the lipid A of gram-negative heterotrophic bacteria, suggests that cyanobacterial lipid A is quite different from that of heterotrophic gram-negative bacteria (10, 19).

Healy (7) observed that cultured *A. flos-aquae* UTEX 1444 strain displayed morphological characteristics similar to those of *A. variabilis*. This classification was accepted also by Tison and Lingg (31). Weckesser et al. (35) isolated and analyzed LPS from *A. variabilis* (*Anabaena* PCC7118). However, according to our results, LPS from *A. flos-aquae* UTEX 1444 and *A. variabilis* were substantially different, especially for lipid A. In addition to palmitic acid and three β-hydroxy fatty acids, 10 long-chain saturated and unsaturated fatty acids absent in *A. variabil-*

TABLE 4. Biological characterization of *A. cylindrica*, *A. flos-aquae* UTEX 1444, *O. tenuis*, and *O. brevis*

Cyanobacterial strain	Lethality in mice (mg/mouse)	Schwartzman reaction (μg)	LAL gelation (lowest concn possible) (ng)	Rabbit loop assay (500 μg)
A. flos-aquae UTEX 1444	No death	40 and 80 (Weakly positive)	12.5	Negative
A. cylindrica	LD_{50}, 2.64	ND[a]	1	ND
O. brevis	LD_{50}, 3.83	ND	10,000	ND
O. tenuis	ND	20, 40, and 80 (Negative)	100	ND

[a] ND, Not done.

is LPS were isolated from *A. flos-aquae* (Table 3). This indicates that these two strains may represent separate species.

Differences in chemical composition of LPS are important for taxonomical classification of cyanobacteria (28). A similar characterization has been performed on serotypes of bacteria, such as *Salmonella* sp. and *Escherichia coli*, and led to their division into 40 chemotypes according to the sugar composition of the LPS (14, 22). Similarly, the *Citrobacter* serotypes have been divided into 20 chemotypes by Keleti et al. (16, 17) and Sedlak et al. (26, 27). Until recently, the taxonomy of cyanobacteria has been based primarily on cell morphology and size, but future classifications may depend more on chemical characterization.

The absence of LPS from *A. flos-aquae* NRC 44-1 is consistent with the observation of Wang and Hill (32), who isolated only a polysaccharide from another strain of *A. flos-aquae* A37. They hypothesized that the occurrence of LPS among cyanobacteria cannot be generalized. Weckesser et al. (34) have indicated that the cyanobacteria in the chroococcacean subgroup always contain LPS, whereas its presence in other blue-green algae is variable. However, the results of our study and the LPS isolation performed by Mikheyskaya et al. (20), and by Keleti et al. (15) have shown so far that all of the members of the oscillatorian subgroup possess LPS.

The isolated endotoxin from *A. flos-aquae* was nontoxic to mice when injected intraperitoneally. Similarly, Weise et al. (36) have shown that LPS isolated from *Synechococcus* PCC (*A. nidulans*) was not toxic to mice. Mikheyskaya et al. (20) have demonstrated that glucan-free LPS from *Phormidium* spp. was not toxic to mice. It has been previously shown that cyanobacterial LPS are toxic to adrenalectomized mice (13). Here, LPS from *O. brevis* and *A. cylindrica*, when injected intraperitoneally into untreated mice, resulted in LD_{50} values of 3.8 and 2.6 mg per mouse, respectively. These are the first cyanobacterial endotoxins found to cause mortality in untreated mice. LPS isolated from various smooth strains of *Salmonella* sp. have an LD_{50} ranging from 300 to 500 μg per mouse. Thus, experiments with adrenalectomized mice and bioassays with *O. brevis* and *A. cylindrica* LPS suggest that the toxicity of cyanobacterial endotoxins may be approximately 10 times lower than that for heterotrophic gram-negative bacteria (*Salmonella* sp.)

Positive Schwartzman reaction was observed with LPS from *A. flos-aquae*, but not with LPS from *O. tenuis*. All cyanobacterial LPS isolated in our laboratory cause LAL gelation ranging from 1 to 10,000 ng. *Klebsiella* sp. or *E. coli* LPS evoke LAL gelation at concentrations from 0.125 to 2.5 ng. The rabbit loop assay was negative with 500 μg of *A. flos-aquae* LPS 7 h after the injection of LPS into the small intestine.

Generally, all examined biological reactions of cyanobacterial LPS were much weaker than the same tests with endotoxin from gram-negative bacteria.

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